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Phages as Therapeutic Tools to Control Major Foodborne Pathogens: *Campylobacter* and *Salmonella*

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1. Introduction

Foodborne diseases are a growing public health problem worldwide with *Campylobacter* and *Salmonella* being the most common and widely distributed causative agents. These Gram-negative bacteria are common inhabitant of the gut of warm-blooded animals, especially livestock, being transmitted to humans primarily through the consumption of contaminated food of animal origin. Poultry meat and derivatives are regarded as the most common source of human salmonellosis and campylobacteriosis.

In addition to the high prevalence of such pathogens and the consequent health problems caused, control of these pathogens has become increasingly difficult due to the emergence of antibiotic-resistant strains. This emergence is a result of the misuse of antimicrobials in food animals, compromising the action of once effective antibiotics in the treatment of foodborne diseases in humans.

Recent legislation restricting the use of antibiotics as growth promoters in animal production, together with the risk of antibiotic-resistant bacteria entering the human food chain have been the driving force for the development of alternative methods for pathogen control. (Bacterio)phages are naturally occurring predators of bacteria, ubiquitous in the environment, with high host specificity and capacity to evolve to overcome bacterial resistance which makes them an appealing option for the control of pathogens. Several studies have been carried to assess the potential use of phages in the control of *Campylobacter* and *Salmonella* in animals and food material in order to prevent transmission of these pathogens to humans. Overall, although eradication of the target bacteria is an extremely unlikely event, the proof of principle, that phages are able to reduce the number of these pathogens has been established. Even so, some considerations should be taken into account for an efficient application of phages.

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This chapter aims at giving an overview of the two major foodborne pathogens (*Campylobacter* and *Salmonella*), discussing the problems and concerns related to their prevalence and control, focusing mainly on the potential use of phages as an alternative to other control measures. Consequently, the successes and drawbacks of different studies on the use of phages to control *Campylobacter* and *Salmonella* will be explored. Moreover several aspects of phage biocontrol will be addressed. These include considerations on phage characterization, phage dose and route of administration, and ways of overcoming the emergence of phage resistant-bacteria. Finally the requisites for an acceptable phage product and the issues related to its public acceptance will be discussed.

2. Foodborne diseases

Foodborne diseases are of major concern worldwide. The Centres for Disease Control and Prevention (CDC) estimates that 76 million cases of foodborne diseases occur every year in the United States causing roughly 5000 deaths (Nyachuba, 2010). In Australia the number of cases (5.4 million) has been estimated to have an associated cost of 1.2 billion dollars per year (OzFoodNet Working Group, 2009). The European Food Safety Authority (EFSA) reported a total of 5,550 foodborne outbreaks, causing 48,964 human cases, 4,356 hospitalizations and 46 deaths in 2009 (European Food Safety Authority, 2011). While significant attention is usually given to major foodborne outbreaks, studies indicate that outbreaks only account for a small fraction of *Campylobacter* and *Salmonella* infections in humans (European Food Safety Authority, 2009). While a steady decline in the number of cases attributed to *Salmonella* has been observed since 2004, the number of *Campylobacter* infections has remained constant (Figure 1). Campylobacteriosis caused 198,252 confirmed human cases in 2009 with a fatality rate of 0.02 %, continuing to be the most commonly reported zoonosis in the European Union. A total of 108,614 confirmed human cases were attributed to *Salmonella* with a fatality rate of 0.08 % in the same year. Moreover there is a considerable underreporting, and the true number of cases of illness caused by these two pathogens is likely to be 10-100 times higher than the reported number (European Food Safety Authority, 2011).

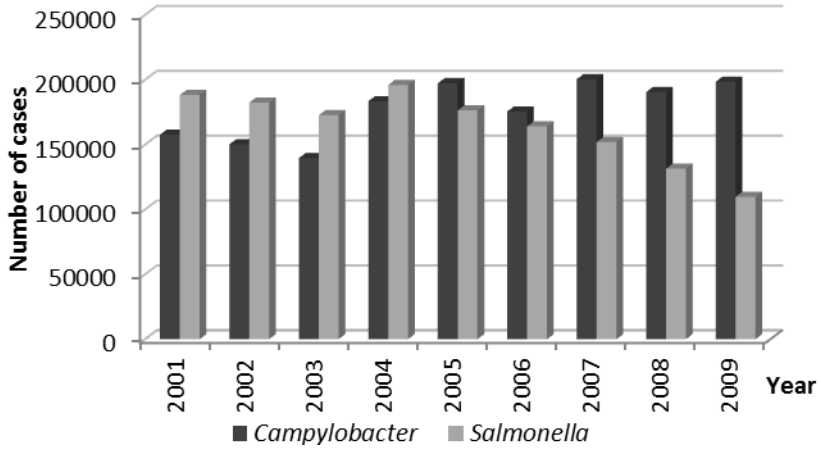

Fig. 1. Number of reported campylobacteriosis and salmonellosis cases in humans 2001-2009 (European Food Safety Authority, 2006; European Food Safety Authority, 2011)

2.1 The pathogens

2.1.1 *Campylobacter*

Campylobacter was first described in 1880 by Theodore Escherich and belongs to the Epsilonproteobacteria, in the order *Campylobacterales* which includes *Helicobacter* and *Wolinella* (Friedman *et al*., 2000; Keener *et al*., 2004). The name *Campylobacter* is derived from the Greek "kampulos" = curved and "bacter" = rod. In fact, bacteria belonging to the genus *Campylobacter* are non-spore forming, oxidase-positive, Gram-negative, curved or spiral (occasionally straight) rods with 0.2 μm to 0.8 μm wide and 0.5 μm to 5 μm long. When they form short or long chains they can appear as S-shaped, V-shaped or more rarely comma shaped. *Campylobacter* ssp. usually displays a long unsheathed polar flagellum at one (polar) or both (bipolar) ends of the cell which confer to this microorganism a rapid, darting and reciprocating motility (Keener *et al*., 2004). *Campylobacter* spp. cells tend to form coccoid and elongated forms on prolonged culture or upon exposure to oxygen (Moran & Upton, 1987). These cells may be associated to a viable but not culturable state (VBNC). However the association between cell culturability and cell morphology remains controversy (Keener *et al*., 2004).

The genus *Campylobacter* now comprises 17 member species. The most commonly isolated are *C. jejuni ssp. jejuni, C. coli* and *C. lari* which are referred as thermophilic species. They can grow at 37ºC to 42ºC with a pH in the range of 4.9 to 9.0, but their optimum growth conditions include a temperature of 42ºC and a pH of 6.5 to 7.5. It is known that they cannot multiply below 30ºC and that they require a microaerobic atmosphere (approximately 5% oxygen and 10% carbon dioxide) (Butzler, 2004).

Contrary to most bacteria, *Campylobacter* species do not obtain their energy from the metabolism of carbohydrates but instead from amino acids or tricarboxylic acid cycles intermediates. *C. jejuni* hydrolyzes hippurate and indoxyl metabolizes acetate and reduces nitrate (Butzler, 2004).

2.1.2 *Salmonella*

Investigations on the etiologic agent of the "swine plague" led Theobald Smith, in 1885, to the isolation of a Gram-negative bacillus named *Bacterium suipestifer*. The bacterium was further characterized by D. E. Salmon from whom the name *Salmonella* is derived. The non-spore forming cells possess a straight rod-shaped morphology with sizes varying from 0.7 μm to 1.5 μm in diameter and 2 μm to 5 μm in length. These cells are usually motile presenting peritrichous flagella. *Salmonella spp.* belong to the *Enterobacteriaceae* family and are chemoorganotrophs (organisms which use organic compounds as their energy source), facultative anaerobes and hydrogen sulphide producers (Bell & Kyriakides, 2002).

The outer membrane (OM) of *Salmonella*, as with almost all Gram-negative bacteria, is composed of OM proteins (OMPs) and lipopolysaccharides (LPS). LPS plays an essential role in maintaining the cell structural integrity and protection from chemicals. In the host organisms they act as endotoxins and as a pyrogen displaying a strong immune response. Structurally they are composed by three distinct components: lipid A, core oligosaccharide and O-polysaccharide (Raetz & Whitfield, 2002). The O-polysaccharide (also O-antigen or O-side-chain) together with the H-antigen (from flagella) and Vi (capsular antigens) are the basis for the Kauffman-White classification scheme, enabling the different *Salmonella* to be

grouped in serotypes according to their agglutination pattern when reacted with specific commercial antisera (Bell & Kyriakides, 2002; Brenner *et al*., 2000). This classification led to the recognition of more than 2500 serotypes (Bell & Kyriakides, 2002), a number that increases every year. A revision of the nomenclature has established two species (*S. enterica* and *S. bongori*) with the majority of the serotypes grouped into one of the six *Salmonella* subspecies of *S. enterica* (Bell & Kyriakides, 2002; Brenner *et al*., 2000; Velge *et al*., 2005).

2.2 The diseases

Campylobacter and *Salmonella* are common inhabitant of the gut of warm-blooded animals mainly livestock (such as cattle, sheep, pigs and chickens), domestic pets and wild animals, where they asymptomatically colonize and multiply (Antunes *et al*., 2003; Bell & Kyriakides, 2002; Bryan & Doyle, 1995; Doyle & Erickson, 2006; Newell & Fearnley, 2003). As zoonotic agents, *Campylobacter* and *Salmonella* can be transferred between humans and other animals. The common route of these pathogens is the consumption of contaminated food of animal origin, particularly meat from pigs, cattle and poultry (and derivatives) and milk. Poultry and derivatives are repeatedly pointed out as the most common sources of infection since the pathogens are present at a high level in fresh poultry meat. *Campylobacter* and *Salmonella* strains may also reach humans via routes other than food, directly by the contact with contaminated animals, carcasses or the environment, for example, through drinking water (European Food Safety Authority & European Centre for Disease Prevention and Control, 2011). Therefore, horizontal transmission appears to have a major role in the transmission of these foodborne pathogens. In contrast to *Salmonella*, vertical transmission of *Campylobacter* is generally considered a relatively unimportant route of flock colonization with the consequent general absence of *Campylobacter* in eggs, one of the most common routes of contamination by *Salmonella* (European Food Safety Authority, 2011; Newell & Fearnley, 2003).

These microorganisms have the ability to survive for considerable periods, especially in conditions that are moist, cool and out of direct sunlight. As a result, they can readily contaminate other hosts, as for example, humans where *Campylobacter* infection is usually associated with illness and for which doses as low as 500 organisms have been reported to cause gastrointestinal disorders (Friedman *et al*., 2000; Newell & Fearnley, 2003; Robinson, 1981). As a consequence, bird-to-bird transmission within flocks is very rapid and it was demonstrated that once a broiler flock becomes infected with *Campylobacter*, close to 100% of birds are reported to become colonized in a very short time (Allen *et al*., 2007; Newell & Fearnley, 2003). Moreover it is known that, after in vivo-passage, organisms can exhibit an enhancement of colonization potential of at least 1,000-fold in most strains and up to 10,000-fold in some strains (Berndtson *et al*., 1992; Keener *et al*., 2004). The most important *Campylobacter* species associated with human infections are *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* (European Food Safety Authority, 2011; Friedman *et al*., 2000; Robinson, 1981). *Campylobacter* has become the most recognized antecedent cause of Guillain-Barré syndrome (GBS), an acute post-infectious immune-mediated disorder affecting the peripheral nervous system that can be permanent, fatal or last several weeks and usually requires intensive care (Butzler, 2004; Nachamkin, 2002).

The factors contributing for the high prevalence of these pathogens in poultry meat are bad slaughter conditions, cross-contamination, inadequate heat treatment, raw meat and

inappropriate food storage (European Food Safety Authority, 2011; Gorman *et al.*, 2002; Hansson *et al.*, 2005; Jacobs-Reitsma, 2000; Johannessen *et al.*, 2007; Luber *et al.*, 2006; van de Giessen *et al.*, 2006). A European Union-wide baseline survey on *Campylobacter* demonstrated that in EU 71.2% of broilers are colonized by *Campylobacter* at the slaughterhouse (European Food Safety Authority, 2010). Therefore, controlling *Campylobacter* and *Salmonella* infections has become an important goal particularly for the poultry industry.

Human infection by these pathogens results in a gastrointestinal infection, which is usually characterized by an inflammatory reaction, watery (sometimes bloody) diarrhoea, fever, vomiting, abdominal cramps and dehydration which can become severe and life-threatening as a result of tissue invasion and toxin production (Bell & Kyriakides, 2002; Butzler, 2004; Friedman *et al.*, 2000; Nachamkin, 2002; Uzzau *et al.*, 2000). *Salmonella* infections are influenced by the bacterium's host range or degree of host adaptation, enabling the division of the bacteria in two groups: host adapted and ubiquitous. The higher the adaptation of *Salmonella* to a host, the higher the pathogenicity, with a consequent severity of the disease, usually leading to septicaemia (Bell & Kyriakides, 2002; Uzzau *et al.*, 2000; Velge *et al.*, 2005). The most prevalent and important *Salmonella enterica* serotypes reported worldwide are Enteritidis and Typhimurium. These are responsible for 99% of salmonellosis in humans and warm-blooded animals (Bell & Kyriakides, 2002; Brenner *et al.*, 2000).

2.3 Antibiotic resistance

Antibiotics were introduced in the 1940s and have been widely used in the United States (US) and Europe (EU) in livestock and poultry since the 1950s. In the US at least 17 antimicrobials were approved to be used in food animals. In Europe, all classes of antibiotics licensed for human medicine were allowed for use in animals. As a consequence, antibiotics were used in food animals therapeutically, prophylactically and as food supplements to promote faster growth by improving feed efficiency. The discovery of antibiotics growth-enhancing effect became an important element of intense animal husbandry leading to their increased use, often in sub-therapeutic doses in healthy animals and without veterinary prescription (Castanon, 2007; Mathew *et al.*, 2007; Sapkota *et al.*, 2007; World Health Organization, 2002).

The amount of antibiotics used in the absence of disease for non-therapeutic purposes in livestock far exceeds the amount of antimicrobials used in human medicine. It was estimated that 60 to 80% of the antibiotics produced in the US is used for this purpose. The use of antibiotics in livestock has become a major source of concern because of the possibility that they contribute to the declining efficacy of antibiotics used to treat bacterial infections in humans (Smith *et al.*, 2002). This may happen because antimicrobial agents used for food-producing animals are frequently the same or belong to the same classes as those used in human medicine. The later includes tetracyclines, macrolides and fluoroquinolones (Aarestrup *et al.*, 2008; Mellon *et al.*, 2001; Sapkota *et al.*, 2007).

Two EU agencies, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), reported recently on the high incidence of antibiotic resistance in *Salmonella* and *Campylobacter,* and stated their concern. In fact, the high percentage of *Salmonella* and *Campylobacter* isolates displaying resistance to ciprofloxacin is alarming since it represents one of the drugs of choice in human treatment.

High resistance of *Salmonella* to tetracyclines, ampicillin and sulphonamides was also reported, as was *Campylobacter* resistance to high levels of tetracyclines. The EFSA report concluded that the animal antimicrobial usage might be an important factor accounting for the high proportion of resistant isolates (European Food Safety Authority, 2011; European Food Safety Authority & European Centre for Disease Prevention and Control, 2011; Gyles, 2008; Rabsch *et al.*, 2001).

The resistance problem has led the World Health Organization (WHO) in 2002 to advise and encourage all countries to reduce the use of antibiotics outside human medicine and has already established some measures in the surveillance of foodborne diseases in order to reduce the emergence of resistant bacteria with special concern for *Salmonella* and *Campylobacter* (Smith *et al.*, 2005; World Health Organization, 2002). This concern was already present in the EU where several countries have banned the use of antimicrobials that are used in human medicine as growth promoters. The consequent reduction of the selective pressure, has already resulted in a reduction of antimicrobial resistance in a national population of food animals (Aarestrup *et al.*, 2001; Castanon, 2007; Emborg *et al.*, 2003; Smith *et al.*, 2005; Swann, 1969; Tacconelli *et al.*, 2008; Wierup, 2001; World Health Organization, 2002).

Antibiotics are usually the last resource in pathogens control leaving no hope in the treatment of multiresistant bacteria for which no effective antimicrobial exists. Consequently, it can be concluded that an efficient alternative to antibiotics is critical and urgent. In order to control foodborne pathogens, the poultry industry decontaminates carcasses using both chemical and physical treatments. Chemical treatments include washing of carcasses in electrolyzed or chlorinated water, dipping carcasses in a solution containing acidified sodium chlorite before chilling, immersion in acetic or lactic acid or in sodium triphosphate solutions. Physical treatments include freezing of contaminated carcasses, heat-treatment of fresh broiler carcasses, dipping of fresh carcasses in hot water immediately before chilling, radiation, exposure to dry heat, and ultrasonic energy in combination with heat (Corry & Atabay, 2001; Keener *et al.*, 2004). In spite of the effort that has been done to control these pathogens, they are still a major cause of foodborne diseases (European Food Safety Authority, 2011; Nyachuba, 2010)(Figure 1).

Other possible control measures to eliminate or reduce the contamination of birds are still being developed and their cost-effectiveness and applicability to large-scale production remain to be determined. It includes: vaccination, the use of competitive exclusion, improving genetic resistance of birds and the use of probiotics, bacteriocins and bacteriophages (Chen & Stern, 2001; García *et al.*, 2008; Joerger, 2001).

3. Bacteriophages: Novel therapeutic agents

(Bacterio)phages are viruses that are able to infect Bacteria. Phages are able to infect more than 150 bacterial genera, including aerobes and anaerobes, exospore and endospore formers, cyanobacteria, spirochetes, mycoplasmas, and chlamydias (Ackermann, 2001; Ackermann, 2009).

Structurally they consist of a nucleic acid genome enclosed within a protein or lipoprotein coat and like all viruses are absolute parasites, inert particles outside their hosts, deprived of their own metabolism. Inside their hosts, phages are able to replicate using the host cell as a

factory to produce new phages particles identical to its ascendant, leading to cell lysis and consequent death of the host (Guttman *et al.*, 2005). As a result of their bacterial parasitism, phages can be found wherever bacteria exist and have already colonized every conceivable habitat. Phages are an extremely diversified group and it has been estimated that ten phage particles exist for each bacterial cell. This fact accounts for an estimated size of the global phage population to be approximately 10^{31} particles making phages the most abundant living entities on earth (Rohwer, 2003).

Their presence in the biosphere is especially predominant in the oceans presenting an excess of 10^7 to 10^8 phage particles per millilitre in coastal sea and in non-polluted water and comparably high numbers in other sources like sewage and faeces, soil, sediments, deep thermal vents and in natural bodies of water (Rohwer, 2003). In the absence of available hosts to infect, and as long as they are not damaged by external agents, phages can usually maintain their infective ability for decades (Guttman *et al.*, 2005; Sharp *et al.*, 1986).

3.1 Phage therapy versus chemotherapy

Phage therapy presents many potential advantages over the use of antibiotics which are intrinsic to the nature of phages. Phages are highly specific and very effective in lysing the target pathogen, preventing dysbiosis, that is, without disturbing the normal flora and thus reducing the likelihood of super-infection and other complications of normal-flora reduction that can often result following treatment with chemical antibacterials. This high specificity means that diagnosis of the bacteria involved in the infection is required before therapy is employed (Guttman *et al.*, 2005; Marks & Sharp, 2000; Matsuzaki *et al.*, 2009). The specificity of phages also enables their use in the control of pathogenic bacteria in foods since they will not harm useful bacteria, like starter cultures. Moreover, phages do not affect eukaryotic cells, or cause adverse side effects as revealed through their extensive clinical use in the former Soviet Union. Furthermore, phages are equally effective against multidrug-resistant pathogenic bacteria.

It was also found that phages can rapidly distribute throughout the body reaching most organs including the prostate gland, bones and brain, that are usually not readily accessible to drugs and then multiply in the presence of their hosts (Dabrowska *et al.*, 2005). The self-replicating nature of phages reduces the need for multiple doses to treat infection diseases since they will replicate in their pathogenic host increasing their concentration over the course of treatment leading to a higher efficacy. This also implies that phages will be present and persist at a higher concentration where their hosts are present, which is where they are more needed, in the place of infection. Reciprocally, where and if the target organism is not present the phages will not replicate and will be removed from the system showing the other side of the self-replicating nature of phages, their self-limiting feature (Goodridge & Abedon, 2003; Petty *et al.*, 2007).

As it happens with antibiotics, bacteria also develop resistance to phages. The latter usually occurs through loss or modification of cell surface molecules (capsules, OMPs, LPS, pili, flagella) that the phage uses as receptors. Since some of these also function as virulence determinants their loss may in consequence dramatically decrease the virulence of the bacterium or reduce its competitiveness (Levin & Bull, 2004). A good example is that of Smith (1987) that used phages against the K1 capsule antigen of *Escherichia coli* and verified

that resistant K1 bacteria were far less virulent (Smith *et al*., 1987b). Furthermore, different phages binding to the same bacteria may recognize different receptors and resistance to a specific phage does not result in resistance to all phages. Phages are able to rapidly change in response to the appearance of phage-resistant mutants making them efficient in combating the emergence of newly arising bacterial threats (Matsuzaki *et al*., 2009; Sulakvelidze *et al*., 2001). In addition, the isolation of a new phage able to infect the resistant bacteria can be easily accomplished. It is much cheaper, faster and easier to develop a new phage system than a new antibiotic which is a long and expensive process (Petty *et al*., 2007).

4. Phage potential in food safety

Phages can be used to combat pathogens in food at all stages of production in the classic 'farm-to-fork' continuum in the human food chain (García *et al*., 2008). Accordingly, in order to prevent transmission to humans, phages can be used:

i. in livestock to prevent diseases or reduce colonization;
ii. in food material (such as carcasses and other raw products) or in equipment and contact surfaces to reduce bacterial loads;
iii. in foods as natural preservatives to extend their shelf life.

Several studies have been carried to assess the potential use of phages in the control of *Campylobacter* and *Salmonella* in animals and foodstuff. Although very different results have been obtained it seems that the proof of principle has been established: phage therapy has potential in the control of foodborne pathogens (Johnson *et al*., 2008). The large scale, high throughput and mechanization of poultry production and industry, made poultry and products the most commonly used models for phage biocontrol (Atterbury, 2009). This is reflected in the studies that will be addressed below.

4.1 *Campylobacter* and *Salmonella* phages

4.1.1 *Campylobacter*

There are relatively few reports on *Campylobacter* phages probably due to the fastidious growth conditions of their host and to unique features that their phages exhibit. This has hindered the use of conventional methods of phage isolation, propagation and characterization (Bigwood & Hudson, 2009; Tsuei *et al*., 2007). Recently Carvalho *et al.* (2010) described an improved method for *Campylobacter* phage isolation in which a pre-enrichment of the phages with potential host strains supplemented with divalent cations was used to promote phage adherence to the host (Carvalho *et al*., 2010b). *Campylobacter*-specific phages have been isolated from excreta of both broiler and layer chickens, retail poultry, and other sources including pig, cattle and sheep manure, abattoir effluents, and sewage (Connerton *et al*., 2011). Some of these phages have been characterized and form the basis of the United Kingdom phage typing scheme (Frost *et al*., 1999; Sails *et al*., 1998).

The most frequently encountered *Campylobacter* phages belong to *Caudovirales* order, *Myoviridae* family with a double-stranded DNA genome enclosed in icosahedral heads (Connerton *et al*., 2008). *Campylobacter* phages have been characterized into three groups according to their genome size and head diameter (Sails *et al*., 1998): Group I - head diameters of 140 nm – 143 nm and large genome sizes of 320 kb; Group II - average head

diameters of 99 nm and average genome sizes of 184 kb; Group III - average head diameters of 100 nm and average genome sizes of 138 kb. Hansen *et al.* (2007) characterized *Campylobacter* phages according to their genome size and susceptibility of digestion by HhaI (Hansen *et al.*, 2007).

The DNA of most *Campylobacter* phages is difficult to extract, clone and sequence and is refractory to restriction enzyme digestion, which is probably due to tightly adherent and proteinase K resistant proteins (Carvalho *et al.*, 2011; Hammerl *et al.*, 2011; Kropinski *et al.*, 2011; Timms *et al.*, 2010). As a consequence, the genome sequence of only five *Campylobacter* phages have been reported so far (Carvalho *et al.*, 2011; Hammerl *et al.*, 2011; Kropinski *et al.*, 2011; Timms *et al.*, 2010). Interestingly, the phage genomes known are all related and also part of the T4 superfamily of phages (Petrov *et al.*, 2010).

There is little information available regarding the prevalence and influence of phages on *Campylobacter*-colonized poultry flocks. In fact, the prevalence of *Campylobacter* phages in poultry has essentially only been described in the UK. It was reported that *C. jejuni* phages were isolated from 20% of the caeca of chickens sampled in which there was a correlation between the presence of natural environmental phages and a reduction in the numbers of colonizing *Campylobacter*. Interestingly, birds that harbored phages had a significant difference ($P < 0.001$) in *Campylobacter* colony forming units (CFU) per gram in relation to those that did not have phages (Atterbury *et al.*, 2005). *Campylobacter* phages were prevalent in the caecal contents of organic birds with 51% of *Campylobacter*-positive sampled birds also carrying phages. The higher value of phage positive samples in organic flocks can be explained by the fact that these birds are more exposed to the environment and therefore to a greater range of *Campylobacter* types and phages (El-Shibiny *et al.*, 2005).

It was also showed that, like *Campylobacter*, their phages are also transferred between flocks (Connerton *et al.*, 2004). Moreover, phages were also recovered from chilled retail poultry, meaning that these phages can survive on retail chicken under commercial storage condition (Atterbury *et al.*, 2003b).

4.1.2 *Salmonella*

Numerous phages infecting *Salmonella* have been isolated. The first report of a *Salmonella* phage dates back to 1918 and was described by Félix d'Hérelle. Since then, *Salmonella* phages have been isolated from different sources: wastewater plants, sewage, manure, faeces and caecal contents from different animals (e.g. poultry, turkey, pig, humans), zoo ponds, nests from poultry farms and many others (Andreatti Filho *et al.*, 2007; Santos *et al.*, 2010; Sillankorva *et al.*, 2010). The search for different *Salmonella* phages from different sources may be attributed to the interest prompted by the medical and veterinary significance of their pathogenic host.

The great number and different specificity of *Salmonella* phages has enabled *Salmonella* classification through phage typing, a useful typing tool for subcategorizing the more common *Salmonella enterica* serotypes (i.e. *S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg) recommended by the WHO.

Probably the best known *Salmonella* phages are the lytic phage Felix 01 and the temperate virus P22. Felix 01 is characterized by its broad lytic spectrum among *Salmonella* and has

been used as a diagnostic tool in the identification of *Salmonella*. Recently, a phage with a broader host range than Felix 01 has been described which presents great potential not only as a therapeutic agent but also as a diagnostic tool (Santos *et al.*, 2010; Santos *et al.*, 2011; Sillankorva *et al.*, 2010) (Figure 2).

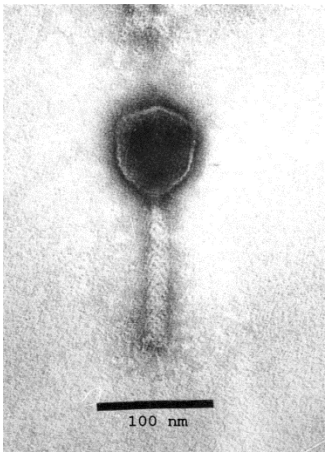

Fig. 2. TEM observation of the broad host range myovirus *Salmonella* phage PVP-SE1 (Santos *et al.*, 2010)

A survey conducted by Ackermann identified 177 *Salmonella* phages of which 91% belong to the *Caudovirales* order. Their distribution by families is roughly equal with 24% *Myoviridae*, 31% *Siphoviridae* and 33% *Podoviridae*. The 9% left are distributed in the *Inoviridae*, *Leviviridae*, *Microviridae*, and *Tectiviridae* families (Ackermann, 2007). A phylogenetic analysis relying on a proteomic comparison resulted in the recognition of at least five groups: P27-like, P2-like, lambdoid, P22-like, and T7-like. Nevertheless, three *Salmonella* phages (epsilon15, KS7, and Felix O1) are outliers since they could not be attributed to any of the previous groups (Kropinski *et al.*, 2007).

4.2 Phage biocontrol in livestock

As already outlined above, contamination of meat products with *Campylobacter* and *Salmonella*, as well as other foodborne pathogens, often results from cross-contamination between carcasses and feces from infected animals during slaughter and processing and also during transportation, leading to an increase of bacterial loads. Phage biocontrol in livestock presents two major purposes: i) treatment of bacterial pathogens in animals to minimize its impact on animal health and production and, ii) control of foodborne pathogens contamination to humans through foodstuff or other vectors. Therefore, the use of phages to control pathogens in livestock seems to be a feasible and efficient approach.

In this chapter the term phage therapy will refer only to the use of phages to control bacterial infections in living animals, whereas, the term biocontrol will be used where phages control pathogens in animals and foodstuff (independent of infection) (Hagens & Loessner, 2010).

4.2.1 *Campylobacter*

There are only a few reports on phage biocontrol against *Campylobacter*-infected livestock, with all the studies being conducted on poultry. Wagenaar *et al.* (2005) assessed both preventive and therapeutic phage treatment (Wagenaar *et al.*, 2005). In their study, multiple doses of a group III phage were administered to chicks before or after being orally challenged with a *C. jejuni* strain. In order to access the effect of phage administration on broilers, the *Campylobacter* colony forming units (CFU) and the phage plaque forming units (PFU) from caecal contents were enumerated throughout the experimental period. These values were obtained from the group receiving phages and from the control group (in which birds did not received phages). In both treatments, birds were orally challenged with a dose of $1X10^5$ CFU *C. jejuni* ten days after hatching. Preventive treatment consisted in the oral administration of phage 71 ($4X10^9$ to $2X10^{10}$ PFU) for ten consecutive days, starting seven days after hatching. The phage treatment did not prevent the colonization of the caecum, but may had delayed it. In fact, initially the numbers of *Campylobacter* were reduced by 2 $\log_{10}$ CFU/g but after one week the numbers leveled out at approximately 1 $\log_{10}$ below that of the controls. In the therapeutic treatment the phage was orally administrated five days after birds being challenged with *C. jejuni* and consecutively during the next six days. The numbers of *Campylobacter* had decreased 3 $\log_{10}$ CFU/g at 48h, but after five days stabilized to approximately 1 $\log_{10}$ CFU/g below the control group. In order to mimic the "farm condition" in which birds are normally slaughter at 42 days, birds were orally challenged with a dose of $1X10^5$ CFU *C. jejuni* at 32 days after hatching. Seven days later phages 71 and 69 were orally administered to these birds and for four consecutive days. As occurred with the previously described treated group, the values of *Campylobacter* counts dropped initially 1.5 $\log_{10}$ CFU/g but then stabilized at 1 $\log_{10}$ unit lower than in the controls.

In the study by Loc Carrillo *et al.* (2005), broiler chicks at 20 to 22-day-old were challenged with *C. jejuni* strains isolates HPC5 and GIIC8 from United Kingdom broiler flocks that have proved to be good colonizers (Loc Carrillo *et al.*, 2005). The chicks received four different doses of HPC5 (2.7 $\log_{10}$ CFU, 3.8 $\log_{10}$ CFU, 5.8 $\log_{10}$ CFU and 7.9 $\log_{10}$ CFU) and after 48h the *Campylobacter* numbers in the caeca, upper and lower intestine were enumerated. The highest dose led to more reproducible colonization levels of all intestinal sites examined, with a mean value of 6.3 $\log_{10}$ CFU/g, 6.7 $\log_{10}$ CFU/g and 7.4 $\log_{10}$ CFU/g in the upper intestine, lower intestine and caeca respectively. Moreover these colonization levels, which are similar to those of naturally colonized birds (Rosenquist *et al.*, 2006), were maintained over nine days. The phage treatment occurred five days after the *C. jejuni* challenge. Birds were treated orally with two phages (CP8 or CP34) independently at a dose of 10^5, 10^7 or 10^9 PFU. The phages were administered in an antacid suspension ($CaCO_3$) since it proved to protect phages from exposure to low pH during passage through the gastrointestinal tract. The administration of 10^7 PFU was the dose which led to the highest reduction (3.9 $\log_{10}$ CFU/g) in the upper and lower intestine and caecal counts of *Campylobacter* at 24 h. The highest dose was less effective in the treatment probably due to the aggregation and nonspecific association of phages with digesta, non-host bacteria or bacterial cell debris resulting from "lysis from without" (Rabinovitch *et al.*, 2003). Different host-phage combinations were tested *in vivo*, leading to different results. In fact, and contrarily to the results obtained *in vitro*, phage CP34 was more effective in the reduction of *C. jejuni* HPC5 at all intestinal sites compared to CP8. Conversely phage CP8 was efficient in the reduction of *Campylobacter* counts by more than 5 $\log_{10}$ CFU/g in caecal *Campylobacter* counts. Despite

this initial reduction, the *C. jejuni* numbers started to increase 72 h after phage administration with the lower intestinal counts exhibiting significant differences of 2.1 $\log_{10}$ and 1.8 $\log_{10}$ CFU/g but with the upper intestinal counts showing no significant difference with the *Campylobacter* levels recorded for the control group.

El-Shibiny *et al.* (2009) reported the administration of a group II *Campylobacter* phage (CP220) to *C. coli* and *C. jejuni* colonized chickens (El-Shibiny *et al.*, 2009). The results showed that a 2 $\log_{10}$ CFU/g reduction in *Campylobacter* counts was observed when a single 10^7 or 10^9 PFU dose of CP220 was administered to *C. jejuni* or *C. coli* colonized chickens, respectively. After this treatment, only 2% of the recovered *Campylobacter* displayed resistance to CP220.

Recently, Carvalho *et al.* (2010) tested a phage cocktail composed by three group II *Campylobacter* phages (Figure 3) in chicks that were previously challenged with *C. coli* strain A11 or *C. jejuni* strain 2140 (Carvalho *et al.*, 2010a). Again, colonization models were established before phage therapy experiments were performed in order to access the effective reduction in *Campylobacter* numbers. In order to determine the optimum dose of *Campylobacter* that should be given to birds, the animals were challenged with three different concentrations (10^4, 10^6 or 5.5×10^7 CFU) of an overnight culture of *C. jejuni* and sampling points were obtained during seven days. The results obtained revealed that the dose of *Campylobacter* appeared to have little effect on the outcome of subsequent colonization and that the mean level of colonization was 2.4×10^6 CFU/g, which is within the range of the infection levels found in commercial broilers (Rosenquist *et al.*, 2006). Seven days post-infection, a single dose of a phage cocktail was administered to chicks by two different routes: oral gavage and incorporated into their feed. Sampling points were taken for seven days after phage administration and showed that the phage cocktail was able to reduce by approximately 2 $\log_{10}$ CFU/g the titre of both *C. coli* and *C. jejuni* in faeces of colonized chickens. This reduction persisted throughout the experimental period and none of the pathogens regained their former numbers. The administration in feed led to an earlier and more sustainable reduction of *Campylobacter* than administration by oral gavage. The phage titers from faecal samples of the chicks infected with *Campylobacter* remained approximately constant throughout the experimental period showing that phages delivered to chicks (either by oral gavage or in feed) were able to replicate and therefore able to reduce the *Campylobacter* populations.

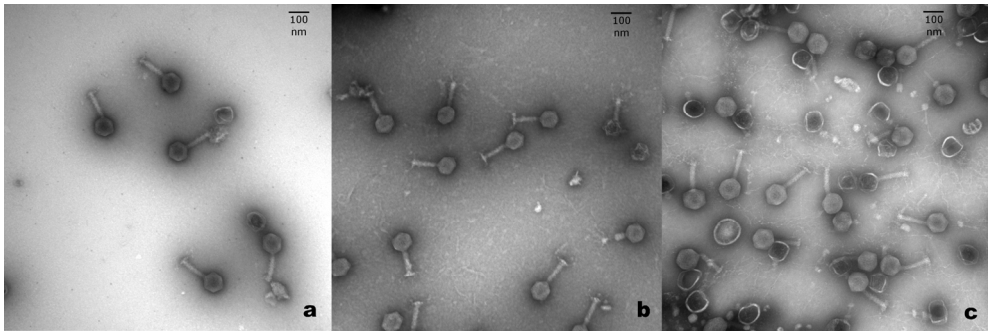

Fig. 3. TEM observation of the three *Campylobacter* phages, belonging to the family *Myoviridae,* which make up the cocktail used in the *in vivo* experiments by Carvalho *et al.* (2010): a) phiCcoIBB12, b) phiCcoIBB35, c) phiCcoIBB37 (Carvalho *et al.*, 2010a)

The appearance of phage resistant mutants has always been seen as a major drawback of phage therapy. Nevertheless some of the above mentioned studies found that phage resistance can be associated with a reduced colonization potential in the chicken intestine, suggesting that there is a fitness cost to phage resistance in which phage-resistant *Campylobacter* revert to a sensitive phenotype when re-colonizing the chicken intestine in the absence of phage predation. It was also suggested that genomic instability of *C. jejuni* in the avian gut can be seen as a mechanism to temporarily survive phage predation and later competition for resources (Carvalho *et al.*, 2010a; Loc Carrillo *et al.*, 2005; Scott *et al.*, 2007a; Scott *et al.*, 2007b). Conversely, recent studies by Carvalho *et al.* (2010) reported that *Campylobacter* strains resistant to phage infection were recovered from phage-treated chickens at a frequency of 13% and that these resistant phenotypes did not exhibit a reduced ability to colonize the chicken guts and did not revert to sensitive types (Carvalho *et al.*, 2010a).

4.2.2 *Salmonella*

The importance and impact of *Salmonella* has elicited several studies on the use of phages on the control of this pathogen. Although poultry represents the most commonly used models, studies also exist in pigs.

In 1991, Berchieri and colleagues used a chicken model to assess the potential of phages to control *Salmonella* Typhimurium in chickens. In the established model, oral infection with 10^9 CFU of *Salmonella* was fatal in 53% of the chickens. Oral administration of phage at high concentration (greater than 10^{10} PFU/ml), soon after *Salmonella* infection, was able to significantly reduce mortality (from 60% to 3%) as well as the number of pathogens in the alimentary tract. The high dose required for the reduction of *Salmonella* suggests that control by the phage was made by "lysis from without" or by a single cycle of replication. Although pathogen numbers were reduced shortly after phage administration, they increased soon after. Moreover, the phage was only present in the intestine when *Salmonella* was found in numbers above 10^6 CFU/ml (Berchieri *et al.*, 1991). Therefore, an efficient application of the phage required that administration should be right after infection and even so bacterial control was possible only for a short period. This may be attributed to the ability of *Salmonella* to internalize cells thus escaping from phages. As with the *Campylobacter* studies (see above) phage resistant bacteria were also found to present rough morphology and to display less virulence than the wild strain. More than one phage was used in this study and one of these, although lytic *in vitro*, was not effective *in vivo* showing the importance of *in vivo* trials.

Fiorentin *et al.* (2005) used a cocktail of three phages to control *S.* Enteritidis PT4 in broilers. The phage dose administered was also very high (10^{11} PFUs of each phage). In this study phage was administered only seven days post infection and a 3.5 $\log_{10}$ CFU reduction per gram of caecal content was recorded five days later. This level remained for 25 days. It seems here that time of administration was not critical but phage dose was. Although a high dose and a cocktail of phages were used with the purpose of reducing the possibility of phage resistant bacteria emergence, the presence of such cells was not assessed (Fiorentin *et al.*, 2005).

When a similar dose (10^{11} PFUs) was given orally two days after infection, Atterbury *et al.* (2007) were able to achieve a reduction of the caecal colonization of both *S.* Enteritidis and *S.*

Typhimurium in broiler chickens by up to 4.2 $\log_{10}$ CFU/g. Lower doses were ineffective in achieving similar results. A third phage tested in this study against *S.* Hadar did not reduce bacterial counts despite its strong lytic activity *in vitro* against that strain. The emergence of resistant bacteria was here also a reality with the number of phage resistant *Salmonella* increasing with the PFUs administered. This subpopulation of resistant bacteria was able to recolonize the broiler chickens within 72 hours after phage treatment. Interestingly, when these resistant bacteria were used to colonize a new group of chickens, they reverted to their phage-sensitive phenotype (Atterbury *et al.*, 2007).

Borie *et al.* (2008) pretreated ten day old broiler chicks, by coarse spray or drinking water containing a cocktail of three phages 24 hours before administering roughly 10^6 CFUs of *Salmonella* Enteritidis (calculated multiplicity of infection (MOI) of 10^3). After ten days of infection phages were recovered from the intestinal and other organs. A significant reduction in *Salmonella* Enteritidis was obtained at that time for both routes of administration with a reduction of more than 1 $\log_{10}$ CFU/ml in challenged bacterial numbers. This study demonstrates not only that phages are able to reduce *Salmonella* bacterial loads in broiler chickens but also that aerosol spray and drinking water are conceivable routes of administration in the application of a phage product which will surely facilitate application and establishment of phage biocontrol in an industrial environment (Borie *et al.*, 2008). In a later study these authors, using seven days old chicks were able to replicate the aerosol results. Moreover, they showed that phage treatment coupled with competitive exclusion resulted in even better *Salmonella* reductions than each of the treatments alone (Borie *et al.*, 2009).

Toro *et al.* (2005) and coworkers also tested the association of phage therapy and competitive exclusion in the treatment of chickens infected experimentally with *Salmonella*. Phage treatment was given orally and included a cocktail of three different phages with different host ranges. In all treated groups, with phage or competitive exclusion alone or together, a decrease in the *Salmonella* counts was observed with a reduction to marginal levels in the ileum and a six fold reduction in the caeca in the case of the group treated with the phage cocktail. Moreover, there was a marginally improved weight gain in the treated animals. Although both approaches were able to reduce the *Salmonella* counts, unlike the previous study (Borie *et al.*, 2009) a synergistic effect was not observed (Toro *et al.*, 2005).

In 2007 Andreatti Filho and colleagues reported on the isolation and testing of two different phage cocktails (one with 4 phages and another with 45 phages) in the control of *Salmonella in vivo* and *in vitro*. *In vitro* test of these two preparations at concentrations of 10^5 to 10^9 PFU/ml in simulated crop environment resulted in a 1.5 or 5 $\log_{10}$ reduction of *Salmonella* Enteritidis, respectively for the 4-phage and 45-phage cocktails in two hours after treatment. Although the 4-phage cocktail did not produce a reduction at six hours post-treatment, the 45-phage cocktail was able to reduce bacterial counts by 6 $\log_{10}$. This study clearly shows the advantage of a cocktail with a large number of phages, probably enabling complementary host range between phages and broader action in bacteria. Phage cocktails were administered at 10^8 PFU by oral gavage to day-of-hatch chicks infected with $9x10^3$ CFU of *Salmonella* Enteritidis. These showed significant reduction of *Salmonella* recovered from caecal tonsils after 24 hours of treatment but no difference was observed at 48 hours when compared with the control group. Another experiment combined the use of the 45-phage cocktail with a commercial probiotic (and controls with each alone) to treat day-of-hatch

chicks achieving significant reductions in *Salmonella* recovery from caecal tonsils at 24 hours but no additive or synergistic effect was observed when combining both approaches. Once again, phage therapy was only efficient during a short period and no long term protection was observed (Andreatti Filho *et al.*, 2007).

Recently, Wall *et al.* (2010) tested the efficacy of a cocktail to treat *S.* Typhimurium experimentally infected pigs shortly before processing in order to reduce carcass contamination. Administration of the phage at the time of infection resulted in a 2-3 $\log_{10}$ reduction of *Salmonella* colonization (Wall *et al.*, 2010).

O'Flynn *et al.*, (2006) isolated two broad host range *Salmonella* phages (st104a and st104b) which were partially resistant to the porcine gastric juice (pH 2.5). *In vitro* tests showed a reduction of more than 2 $\log_{10}$ in *Salmonella* numbers in just one hour demonstrating their potential in controlling *Salmonella* in pigs by oral administration. Even so, as seen in other studies, for an efficient assessment of their potential as therapeutic agents, *in vivo* tests are needed (O'Flynn *et al.*, 2006).

Target pathogen	Livestock	Results	References
Campylobacter	chicken	Reduction of up to 3 $\log_{10}$ CFU/g in caecal samples	(Wagenaar *et al.*, 2005)
	chicken	Reduction of more than 5 $\log_{10}$ CFU in caecal samples	(Loc Carrillo *et al.*, 2005)
	chicken	Reduction of up to 2 $\log_{10}$ CFU/g in caecal samples	(El-Shibiny *et al.*, 2009)
	chicken	Reduction of up to 2 $\log_{10}$ CFU/g (faster reduction when administrated in feed)	(Carvalho *et al.*, 2010a)
Salmonella	chicken	Reduction from 60% to 3% in chicken mortality	(Berchieri *et al.*, 1991)
	chicken	Reduction of up to 3.5 $\log_{10}$ CFU/g of caecal content	(Fiorentin *et al.*, 2005)
	chicken	Reduction of up to 4.2 $\log_{10}$ CFU of the caecal colonization	(Atterbury *et al.*, 2007)
	chicken	Reduction of up to 1.63 $\log_{10}$ CFU/ml in *Salmonella* recovery from the intestine.	(Borie *et al.*, 2008; Borie *et al.*, 2009)
	chicken	Reduction of up to marginal levels in the ileum and six fold in the caeca in the *Salmonella* counts	(Toro *et al.*, 2005)
	chicken	Reduction of up to 6 $\log_{10}$ in simulated crop environment / 55% in recovery from caecal tonsils of chicks	(Andreatti Filho *et al.*, 2007)
	pig	Reduction of up to 3 $\log_{10}$ reduction of *Salmonella* colonization	(Wall *et al.*, 2010)
	pig	Reduction of up to 2 $\log_{10}$ in *Salmonella* numbers (*in vitro* test)	(O'Flynn *et al.*, 2006)

Table 1. Studies on phage biocontrol in livestock

4.3 Phage biocontrol in food material

Another way that phages may be used to improve food safety is to apply them directly onto raw food products. The practical applicability of this approach may be compromised by the minimum density of host cells that are suggested to be required for phage replication (Payne *et al.*, 2000; Payne & Jansen, 2001). Nevertheless it was demonstrated that phages can be effective biocontrol agents when the population of host cells is as low as 46 CFU/cm^2 (Greer, 1988). These contradictory results may be a consequence of differences in phage/host combinations, in the matrix used, in the presence of non-host decoys or even in the models applied. Therefore, the efficacy of phage-based biocontrol should be determined on a case-by-case basis (Atterbury, 2009).

The application of phages as biocontrol agents has been investigated in a variety of food matrices. Nevertheless most studies focus in poultry products, with *Campylobacter* and *Salmonella* being the most frequently targeted zoonotic pathogens.

4.3.1 *Campylobacter*

Most studies on the use of phages as biocontrol are devoted to pre-harvesting studies. In fact, there are only few studies reporting the efficacy of *Campylobacter* phages in foodstuff.

Atterbury *et al.* (2003) studied the survival of *C. jejuni* and phages on chicken skin at temperatures of 4°C or -20°C. A dose of 10^6 CFU *C. jejuni* NCTC 12662 PT14 was inoculated on the surface of chicken skin and then samples were stored at these two temperatures for a period of one hour to ten days (Atterbury *et al.*, 2003a). The results showed that there was a reduction of 1 or 2 $\log_{10}$ CFU on *Campylobacter* counts for skin stored at 4ºC or -20ºC, respectively. This reduction was lower than the value normally reported for *Campylobacter* cells stored at -20ºC (3 $\log_{10}$ CFU) (Chan *et al.*, 2001), which indicates that chicken skin may have had a protective effect. Phage φ2 at 10^7 PFU was added to chicken skin samples and was show to survive for ten days at 4ºC and at -20 ºC. In order to access whether this phage would attach non-specifically to non-susceptible *Campylobacter* strains at 4ºC, this phage was added to *C. coli* NCTC 12667 at a MOI of 10 for ten days. In fact, *Campylobacter* counts and phage titer did not fall during the experimental period which indicates that a nonspecific adsorption did not occur. Conversely when the same phage was added to its susceptible host, under the same conditions described above, the *Campylobacter* numbers were reduced by 0.8 $\log_{10}$ CFU/ml (Atterbury *et al.*, 2003a).

In another experiment performed by the same authors, phage φ2 and *C. jejuni* NCTC 12662 PT14 were added to chicken skin samples at different MOI ranging from 10^{-3} to 10^5 with samples stored at 4ºC and -20ºC for five days. The results again showed a significant reduction in *Campylobacter* counts when the virus were administered at the highest MOI: a 1.1 $\log_{10}$ to 1.3 $\log_{10}$ CFU reduction in samples stored at 4ºC and a 2.3 $\log_{10}$ to 2.5 $\log_{10}$ CFU reduction for frozen samples. Overall, the treatment that showed the best reduction in *Campylobacter* counts in chicken skin was the one in which high phage titers were applied following by storage of samples at freezing temperatures. Nevertheless, in all the treatments phages were not able to replicate. Furthermore, *Campylobacter* strains recovered after phage treatments were shown to be identical to the inoculated strains and did not display resistance to this phage (Atterbury *et al.*, 2003a).

Goode *et al.* (2003) also demonstrated the reduction in *Campylobacter* levels, after the application of a lytic phage to chicken skin, experimentally contaminated with *C. jejuni*. Chicken portions were initially inoculated with 10^4 CFU/cm^2 of *C. jejuni* strain C222 and then half of the chicken pieces were treated with *C. jejuni* typing phage 12673 at an approximate density of 10^6 PFU/cm^2. The samples were incubated at 4ºC and swabs were taken after 24h. The results obtained show that *Campylobacter* counts were reduced by 1.3 $\log_{10}$ CFU comparing with the control. However, there was even a reduction of approximately 1 $\log_{10}$ CFU in the *Campylobacter* numbers from the non-phage-treated chicken portions, which meant that *Campylobacter* did not survive well on exposed chicken surfaces at 4ºC (Goode *et al.*, 2003).

In 2008 Bigwood *et al.* (2008) reported on an investigation into the use of phage Cj6 against *C. jejuni* inoculated on cooked and raw meat and incubated for 24h at two different temperatures (5ºC and 24ºC) in order to simulate refrigerated and room temperature storage. Experiments were performed using different conditions: low (<100 cells/cm^2) or high (10^4 cells/cm^2) host densities and low (10) or high (10^4) MOIs. When the experimental conditions were set for 5ºC, significant differences were obtained for samples inoculated with high MOI and high host density. A reduction of 2.4 $\log_{10}$ CFU/cm^2 and 1.5 $\log_{10}$ CFU/cm^2 was obtained on cooked and raw meat, respectively. The titer of phages inoculated was also reduced by 10%, after 24h of incubation on cooked meat. When samples were stored at 24ºC, at high MOI and low host density, the reduction in *Campylobacter* counts was not significant. However when samples were inoculated at high MOI and high host density, reduction of 2.8 $\log_{10}$ CFU/cm^2 (after 6h) and 2.2 $\log_{10}$ CFU/cm^2 (after 24h) were obtained on cooked and raw meat, respectively. Nevertheless, *Campylobacter* counts were reduced, even in the untreated samples, which may be explained by their sensitivity to the experimental temperatures. Therefore the results did not allow an accurate assessment of the effective reduction by the phage treatment (Bigwood *et al.*, 2008).

Overall the studies suggest that high MOI values are more effective in the control of *Campylobacter* in foods. From the studies outlined above it is evident that *Campylobacter* are not able to grow and multiply under the conditions found on refrigerated raw meat, which renders unlikely phage replication. Nevertheless even if the phage cannot replicate at that temperatures, when reaching the human intestine, bacteria increases its metabolic activity and phages may eventually attach and lyse the target bacteria, leading to a control effect (Goode *et al.*, 2003).

4.3.2 *Salmonella*

Different approaches have been used to assess the effectiveness of phages to control pathogens in foodstuff. *Salmonella* control in poultry products has been constantly highlighted. Goode *et al.* (2003) also used phages to treat chicken skin experimentally contaminated with *Salmonella* Enteritidis (10^3 CFU/cm^2). Different MOIs were tested and for a MOI=1 the phages were able to replicate increasing their number with the consequent reduction of bacteria by less than 1 $\log_{10}$ in 24 hours. For higher MOIs (10^2 and 10^3) the levels of recovered *Salmonella* were reduced by roughly 2 $\log_{10}$ over 48h. Using even a higher MOI (10^5) to treat a more realistic *Salmonella* contamination level (<10^2 CFUs) no recoverable *Salmonella* was obtained, resulting in the total elimination of the pathogen (Goode *et al.*, 2003).

The use of phages to reduce contamination of pig skin was studied by Hooton *et al.* (2011). In their study on pig skin artificially contaminated with *Salmonella* Typhimurium U288 (the most prevalent serovar found in pigs) at levels of 10^3 CFU, a cocktail of four phages which included Felix 01 was employed at a temperature of 4ºC. Although the application of the phage cocktail at MOI of 1 or less produce little or no reduction, the use of MOI above 10 led to a reduction of the pathogen below detectable levels (Hooton *et al.*, 2011).

The effectiveness of phages to control *Salmonella* in carcasses of broiler chickens and turkeys was tested by Higgins *et al.* (2005). In this study, 10^6 PFU of a phage applied to carcasses was deemed to be inefficient in the removal of *S.* Enteritidis at levels below 10^3. Instead, application of $\geq 10^8$ PFU resulted in a marked reduction in the numbers of carcasses with recoverable *Salmonella*. Higgins *et al.* also used a cocktail composed of 72 different *Salmonella* phages to treat naturally contaminated turkey carcasses. The results are promising showing that the cocktail effectively reduced *Salmonella* recovery from the contaminated carcasses. These studies suggest that a high concentration of phage, preferably a cocktail of different phages, should be used to efficiently treat carcasses contaminated with *Salmonella* (Higgins *et al.*, 2005).

Control of *Salmonella* through the action of phages was also tested in raw and cooked beef by Bigwood *et al.* (2008). The samples were inoculated with *Salmonella* at low or high densities (respectively $<10^2$ or 10^4 CFU/cm^2). Afterwards, phages were added at a MOI of 10^1 or 10^4 and samples incubated at different temperatures to simulate refrigerated and room temperature storage (respectively 5ºC and 24ºC). Phages were able to reduce *Salmonella* counts in up to 2.3 $\log_{10}$ CFU/cm^2 for samples incubated at 5ºC and in more than 5.9 $\log_{10}$ CFU/cm^2 for samples incubated at 24ºC when compared to controls (samples inoculated with *Salmonella* without phage). These results were obtained for both high densities of *Salmonella* (10^4 CFU/cm^2) and phages applied at a high MOI (10^4) with samples incubated for 24h. The reductions were even higher after eight days of incubation. For low *Salmonella* densities the reductions were not significant for the majority of the samples. Interestingly, recovered *Salmonella* cells were found to still be sensitive to phage infection (Bigwood *et al.*, 2008).

The well-known broad host range *Salmonella* phage Felix 01 was used by Whichard *et al.* (2003) to treat chicken sausages contaminated with *S.* Typhimurium DT104 (300 CFU) and a reduction of up to 2.1 $\log_{10}$ in the *Salmonella* levels was obtained (Whichard *et al.*, 2003).

Control of *Salmonella* in foodstuff has not been restricted only to meat and derivates. A study carried by Leverentz and colleagues examined the efficiency of phage to control *Salmonella* on fresh-cut fruit, a rapidly developing industry. Treatment with a phage mixture was able to reduce the numbers of *Salmonella* by nearly 3.5 $\log_{10}$ CFU/g in honeydew melon slices stored at 5 ºC and 10ºC, temperatures at which *Salmonella* can survive or increase up to 2 $\log_{10}$ respectively within a week. At 20ºC, where *Salmonella* loads can increase up to 5 $\log_{10}$, the decrease of *Salmonella* in slices was of 2.5 $\log_{10}$. The reductions obtained were greater than the maximal amount achieved using chemical sanitizers. Although this marked reduction in melons, in apple slices this did not happen. The reason may rely in the lower pH of apples that does not enables phage survival since it was not possible to reisolate phages in the apple 48 hours post treatment while in the melons the phage concentration was stable during this time period (Leverentz *et al.*, 2001a).

Pao *et al.* (2004) used phages to treat sprouting seeds where it was found that *Salmonella* grows during soaking. Treatment with phages could reduce the numbers of *Salmonella* in 1.37 $\log_{10}$ and 1.5 $\log_{10}$ in mustard seeds and in the soaking water of broccoli seeds by using one or two phages respectively (Pao *et al.*, 2004).

Cheese contamination by *Salmonella* was also subject of study on possible biocontrol with phages. Modi *et al.* (2001) following standard procedures made cheddar cheese from raw and pasteurized milk (an important vehicle of *Salmonella* transmission to humans) (European Food Safety Authority, 2011). The cheese was inoculated with 10^4 CFU/ml of *S.* Enteritidis and 10^8 PFU/ml of the *Salmonella* phage SJ2 (Modi *et al.*, 2001). A decrease in *Salmonella* counts by 1 to 2 $\log_{10}$ was observed in raw and pasteurized milk cheeses while in the controls (cheeses made from milks inoculated with only *Salmonella* and no phage) an increase of 1 $\log_{10}$ was observed. After storage of the cheeses for 99 days at 8ºC *Salmonella* was present in the controls at a final concentration of 10^3 CFU/g. In the phage treated cheeses only 50 CFU/g were present in the ones from raw milk and no *Salmonella* was recovered from pasteurized milk cheeses after 89 days.

Composting is a complex process used not only to obtain a nutrient-rich substrate but also to significantly reduce pathogen contamination. Even so, improperly composting may result in *Salmonella* survival and thus constitute a vehicle of *Salmonella* transmission to animals and humans. Heringa *et al.* (2010) used a cocktail of five phages to treat a dairy manure compost inoculated with *S.* Typhimurium and observed a 2 $\log_{10}$ reduction within four hours and 3 $\log_{10}$ reduction within 34 hours compared to the controls (Heringa *et al.*, 2010).

Reduction of *Salmonella* through the action of phages was also investigated in wastewater. Turki *et al.* (2011) isolated three different phages and tested their ability to reduce *Salmonella* in TSB medium at two temperatures (30ºC and 37ºC). Phages were applied at three different MOI (10^0, 10^2 and 10^4) alone or as a cocktail of two or three phages. The three phage cocktail was able to reduce all *Salmonella* cultures at both temperatures when using a high MOI. Although, addition of individual or combination of two phages led to the emergence of phage resistant bacteria. Even so, the use of two phages presented better results than the use of an individual phage. The most important result was the eradication of *Salmonella* from the samples when the three phage cocktail was inoculated in raw wastewater (Turki *et al.*, 2011).

4.4 Considerations

The above studies showed inconsistencies in the ability of phages to act as biocontrol agents of *Campylobacter* and *Salmonella* in livestock. Even so, it seems that the proof of principle, that phages are able to reduce the number of these pathogens (at least in a short period after treatment), has been established.

At a first glance, *in vivo* biocontrol of foodborne pathogens made "on-farm" seems to be a good approach since, theoretically, the problem would be treated on its origin. Ideally, phages would be applied through the use of a sole administration of a low dose of phage. The virulent phage should then amplify at the expense of the target bacteria by repeated cycles of replication leading to the host eradication. The increasing number of the progeny phage would remain in the system for some period of time acting prophylactically in a possible subsequent infection. Although, as shown by several studies, eradication of the target bacteria is an extremely unlikely event in part due to the coexistence of a phage-

Target pathogen	Foodstuff	Results	References
Campylobacter	chicken skin	Reduction of up to 2 log_{10} on frozen samples	(Atterbury *et al.*, 2003a)
	chicken skin	Reduction of up to 2.31 log_{10} CFU/g at 4ºC.	(Goode *et al.*, 2003)
	raw and cooked meat	Reduction of up to 2.8 log_{10} CFU on cooked meat	(Bigwood *et al.*, 2008)
Salmonella	chicken skin	Reduction of up to no recoverable *Salmonella*	(Goode *et al.*, 2003)
	pig skin	Reduction of up to below detectable levels at 4ºC	(Hooton *et al.*, 2011)
	chickens and turkeys carcasses	Reduction of up to 93% reduction in *Salmonella* recovery	(Higgins *et al.*, 2005)
	raw and cooked meat	Reduction of more than 5.9 log_{10} CFU on raw meat	(Bigwood *et al.*, 2008)
	chicken sausages	Reduction of up to 2.1 log_{10} in the *Salmonella* levels	(Whichard *et al.*, 2003)
	fresh-cut fruit	Reduction of up to 3.5 log_{10} in *Salmonella* numbers in honeydew melon slices stored at 5 ºC	(Leverentz *et al.*, 2001b)
	sprouting seeds	Reduction of up to 1.5 log_{10} in *Salmonella* numbers	(Pao *et al.*, 2004)
	cheese	Reduction of up to no recoverable *Salmonella* in pasteurized milk cheeses	(Modi *et al.*, 2001)
	dairy manure compost	Reduction of up to 3 log_{10}	(Heringa *et al.*, 2010)

Table 2. Studies on phage biocontrol in foodstuff

resistant bacterial subpopulation. Eradication is even difficult *in vivo* since the number of *Salmonella* and *Campylobacter* is usually higher in broiler chicken intestines than in the carcasses and derived products. Moreover, the colonization of animals in herds or flocks spreads exponentially and infection may be through direct contact with pens and holding facilities which were used before by infected animals. Therefore, the on-farm treatment may lead to the emergence of phage resistant strains with their consequent spread to all animals in the farm. In addition, repeatedly use of phages may induce production of antibodies that will afterwards neutralize the phages, diminishing their effectiveness (Johnson *et al.*, 2008). The emergence of phage resistant bacteria is a major concern in phage biocontrol and long term studies on the resistance and resistant bacteria should be performed.

The consistent reduction of the target bacteria shortly after phage administration, and the need to avoid the emergence of phage-resistant strains, suggests that phages should be applied shortly before slaughter. Indeed, in the period preceding slaughter, the animal is an epidemiological endpoint and the phages, as well the bacteria, will be removed from the contaminated source. This will prevent the emergence, spread and establishment of phage-

resistant strains and will not have an impact on the farm microbial balance. Moreover this approach constitutes a realistic application of phages enabling the administration of a single dose. This will result in a low number of pathogens during food processing with the consequent decrease of cross-contamination, contributing thus to the consumer safety.

Even so, some considerations should be taken into account for an efficient application of phages. Phages can be administered by different routes that will have impact in the efficacy of the phage action but also economic and practical implications. Obviously, the most practical, and probably economical, route of phage application is through food and drinking water, at least for large scale treatments as is the treatment of *Salmonella* and *Campylobacter* in the poultry industry. These routes of administrations will require further studies on the dose (volume and concentration) that will be incorporated into food or added to water as well the need for a way to protect phages from the low pH of the gastrointestinal tract that was shown to often inactivate the majority of phages (Smith *et al*., 1987a). Protection may be enabled through the simultaneously administration of an antacid or through phage proper encapsulation as tested by Carvalho *et al.* (2010) with a *Campylobacter* phage, phiCcoIBB35 (Carvalho *et al*., 2010a), and Ma *et al.* (2008) with *Salmonella* phage Felix 01 (Ma *et al*., 2008), respectively. Alternatively, higher phage concentrations and/or phage mutants resistant to low pH may be used to increase the efficacy of phage treatment. Intramuscular inoculation seems only feasible to treat a low number and/or animals which represent an added value. Another important consideration is the need for *in vivo* studies for each phage in an appropriate model able to mimic the system in which the particular phage is to be administered since *in vitro* behaviour usually does not reflects the phage behaviour *in vivo*, in part due to the immune system response. This fact has also been often observed in the above studies.

In the case where phage therapy immediately before slaughter is not possible due to inefficiency or impracticability, treatment of meat and foodstuff is also a possible approach. When phages are applied in foodstuff (post-slaughter), as in the case of a pre-slaughter phage treatment, the emergence of phage-resistant is also prevented since the phages and bacterial populations will be removed from the contamination source. Some studies, as described above, have addressed the possibility of using phages in the control of foodborne pathogens in foodstuff although, the majority of the *Salmonella* studies were carried at a temperature that is the optimum for the target bacteria to grow. This may lead to erroneous phage efficacy assessment because phage behaviour is highly dependent on the bacterial physiology which in turn also depends on the temperature. Consequently, a reliable study should be carried at the same temperature and other conditions at which the foodstuff is prepared, processed and/or stored. Commercial storage conditions of carcasses, meat and other foodstuff often include a refrigerated temperature of 4ºC in order to halt bacterial growth. Experiments performed at this temperature with *Campylobacter* and *Salmonella* have shown that a relative high dose (high MOI) of phage is needed probably due to the reduced host growth that will prevent phage replication. Even so, this is not much different from what was observed at higher temperatures or even in studies in livestock where, as it is shown here, a high MOI seems to be a requirement to reduce pathogenic loads. The presented studies have shown that little or no reduction was observed for MOIs of 1 or less suggesting that the therapeutic effect of the phages is passive, without taking advantage of their replicating and self-amplification ability. This passive effect of phages is known as

"lysis from without" in which the adsorption and attachment of many phages to the bacterial surface of a single cell results in lysis without phage replication. The passive effect is corroborated by the results of Bigwood *et al.* (2008) who found that phages did not increase in numbers after reducing the *Salmonella* counts. This passive effect performed by the phages is not impaired at temperatures below the minimum for bacterial host growth as seen in these experiments performed at 4ºC and 5ºC reinforcing phage application in foodstuff at storage conditions (Atterbury *et al.*, 2003a; Bigwood *et al.*, 2008). Moreover, this passive effect seems to be less specific than the active one (where phages replicate inside their host) as has been observed by Goode *et al.* (2003) where *Salmonella* strains resistant to phage through restriction were also eliminated by passive effect of phages as long as the phages were able to attach (Goode *et al.*, 2003). Consequently, treatment with high MOIs, although economically not so attractive due to the need of a higher dose, are able to reduce the emergence of phage-resistant strains.

Another requirement of phage biocontrol is the need for broad host range phages and/or the use of a cocktail. Higher reductions in pathogenic bacteria were consistently obtained in livestock and foodstuff when a cocktail of phages with complementary host ranges and target receptors were used. The advantages of using cocktails and broad host range phages will be discussed below.

Risk analysis modelling have shown that a reduction of 2 $\log_{10}$ in faeces of the slaughtered animal or in chicken carcasses can reduce the risk to consumers in 75% or 30 times respectively, in the incidence of campylobacteriosis associated with chickens consumption (Havelaar *et al.*, 2007; Rosenquist *et al.*, 2003). This 2 $\log_{10}$ reduction was shown in these studies to be a possible, practical and realistic goal.

It can thus be concluded that (by using broad host range phages or cocktails of phages with a broad lytic spectrum, applied at high MOI, complemented with *in vivo* studies) both approaches (phage biocontrol in livestock at pre slaughter and in foodstuff) can be used in order to decrease the number of pathogenic bacteria in the food chain and consequently to reduce the incidence of foodborne diseases caused by *Campylobacter* and *Salmonella*. Although the studies only address the use of these two strategies separately, and because their effectiveness relies in the passive ability of phages to lyse the cells (and thus do not depends on the host concentration and physiological state), it can be argued that both approaches can be used together in order to achieve higher reductions of the pathogenic bacterial loads.

5. Prerequisites for an acceptable phage product

The better understanding of phage biology, infectious process and host-range specificity has been tracing the path by which the problems with early phage research may be partially or totally solved. Therefore the knowledge acquired so far coupled with the awareness of the mistakes committed, should be used to develop phage preparations which should meet several criteria whether they are intended to be applied in foodstuff or more importantly in animals and humans. Newly isolated phages should always be characterized in detail and biocontrol should not be attempted before the biology and genomics of the therapeutic phage is well understood. Consequently, the phage host range, virulence, stability and interaction with both innate and active immune systems should be determined.

5.1 Lytic phages

In order to assure safety of phage therapy and eliminate potential risks of failure or even complications, it is critical that phages used in therapy are strictly lytic and without: transducing capabilities, gene sequences having significant homology with known major antibiotic resistance genes, genes for toxins and genes for other bacterial virulence factors (Carlton *et al.*, 2005). Consequently, it is critical to avoid temperate phages. Reasons for this discrimination involve the fact that the latter phages will not kill all the target bacteria due to their ability to lysogenize them and probably more important, there is a high risk of possessing genes that can render the bacteria more virulent (Los *et al.*, 2010). This can happen because certain temperate phages, such as those associated with *Staphylococcus, E. coli, Salmonella, Corynebacterium* and *Clostridium* actually carry virulence genes. In addition, during the transition between the lysogenic cycle and the lytic cycle, the excision of the prophage DNA may be accompanied with small pieces of the bacterial genome thus producing a specialized transducing phage. In addition, certain viruses such as *Salmonella* phage P22 are generalized transducers that are capable of randomly packaging any part of the host genome. Transducing phages will then transfer the host DNA fragments to newly infected bacteria producing changes in the bacterial genomes through recombination or reintegration. This may produce undesired phenotypic changes in their hosts such as resistance to antibiotics, restriction systems and increased bacterial virulence such as: bacterial adhesion, colonization, invasion and spread through human tissues, resistance to immune defences and exotoxin production (e.g. cholera toxin encoded by *Vibrio cholerae*-phage CTX) (Los *et al.*, 2010; Waldor & Mekalanos, 1996). Strictly lytic phages usually do not pose these risks but should be tested for transducing (Waddell *et al.*, 2009).

5.2 Cocktails and broad-host-range phages

Within a given bacterial pathogen different mutants may exist with different susceptibility for a given phage. Although therapy should always match the phage with the target bacteria there are situations where treatment is urgently need, turning that approach impossible. Moreover, bacterial pathogens may mutate during the treatment time period and become resistant to the phage. This risk is real in part due to the narrow host range of phages. A way to circumvent these problems is by using a cocktail of phages targeting different receptors in the pathogen cell and with cross-resistant lytic activity. Consequently, if a bacterium is, or stays, resistant to one phage it is likely that it would not be resistant to a second phage which is immediately available in the cocktail and that targets a different receptor. This approach has been successfully used by Smith *et al.* (1987) in their *E. coli* diarrhoeal work (Smith *et al.*, 1987b).

An alternative to the phage cocktails is the use of broad host range phages that can be isolated through the use of common ubiquitous receptors in the target bacteria as in the case of phages that recognize TolC. In *Salmonella* this outer membrane protein is involved in the adhesion and invasion of host intestinal epithelial cells (Ricci & Piddock, 2010). Broad host range phages are polyvalent phages capable of infecting across bacterial species or genera and thus able to infect the majority of the target bacteria. These phages present a huge advantage as therapeutic agents and from the biotechnological point of view they are much more attractive because a single phage is far easier to characterize and get approved by the regulatory authorities. The existence of such polyvalent phages in nature is rare and only a

few of them are known. Examples include *Salmonella* phage Felix O1 which infects most *Salmonella* serovars (Kallings, 1967) and *Salmonella* phage PVP-SE1 which presents a lytic spectrum even broader than that of Felix 01 (Santos *et al.*, 2010). Nevertheless these phages may present a drawback if they are able to target other non-pathogenic bacteria causing dysbiosis. Therefore the ideal phage for use as biocontrol agent should have a broad host range among the target pathogen without infecting the commensal flora. Examples of these phages include two virulent coliphages which were able to lyse a high percentage of pathogenic *E. coli* strains of various serotypes whilst showed low lytic ability to lyse non-pathogenic *E. coli* strains (Viscardi *et al.*, 2008). These broad host range phages may also be used, not only as alternatives to cocktails, but also to design new cocktails with broader host ranges and consequently more efficient. Furthermore, phage cocktails and broad host range phages will also combat and prevent the emergence of phage resistant strains. Another possibility would be to engineer phages in order to target numerous receptors. Although very interesting and promising from a scientific point of view, this approach would hardly get acceptance from the food authorities (Kropinski, 2006).

5.3 Genome sequencing

A full knowledge of phage genome sequences is important to ensure that the phage does not carry obvious virulence factors, resistance or lysogeny genes. The identification of gene homologies requires detailed bioinformatics analysis. The latter is essential to evaluate possible complications that might arise during phage therapy. It was suggested that data from phage genome sequences could be used to establish a bank of "safe" therapeutic phages increasing the availability, safety and efficacy of phage therapy (Petty *et al.*, 2007).

Phages that break down the bacterial DNA to recycle bacterial host genome for their own DNA synthesis should be selected for therapy since this will hamper coexistence between phage and host. Such phages usually encode enzymes involved in nucleotide metabolism and the corresponding genes can be identified through sequence analysis (Carlton *et al.*, 2005). Even so, due to the usually high number of genes in the phage genome with unknown function, it is never possible to assure at 100% that no virulence factors, resistance or lysogeny genes exist. Correspondingly, the genome sequencing analysis enables to reject the use of phages for which genes were found to code for virulence, resistance or lysogeny but that does not present any experimental biologic evidence of it, that is, which never have shown to lysogenize a bacterium or increase the bacterium virulence and/or resistance.

5.4 Models for host-phage interaction

Phage therapy experimental design is not straightforward due to the self-replicating nature of phages. This means that for each phage, pharmacokinetic information is required which can be achieved by the determination of the phage infection parameters and by the use of a reliable population dynamic model able to predict the phage-bacteria behaviour. Understanding these dynamics will help the transference of *in vitro* results to *in vivo* predictions, explaining why a phage that replicates extremely well in the target bacteria *in vitro* fails to do it *in vivo*. It is expected that mathematical models may help to design experimental studies of population dynamics by identifying and evaluating the relative contribution of phage and bacteria in the course and outcome of an infection (Levin & Bull, 2004). Therefore, *in vivo* assessment of the phage, in a suitable animal model, should always be accomplished.

5.5 Phage production in a large-scale and storage

The increasing interest in phages as therapeutic or biocontrol agents and the intention of commercially distribute a phage or a phage based product demands a large scale production that is not compatible with the conventional double-agar overlay method. Consequently, production of phages will certainly make use of bioreactors and a control and optimization of phage production will play an important role. Good manufacturing practice requirements demands for the development of methods that ensure phage preparations highly purified, free of bacteria, toxins, pyrogenic substances and other harmful components. Although in the majority of animal studies phages were administered as crude lysates without adverse effects for the animal, the removal of endotoxins, exotoxins and cell debris is very important for the safety of the phage product and also for an easier acceptance by the consumers. An option would be the propagation of phages in a non-pathogenic or in a non-toxin producer host (Santos *et al.*, 2010). The storage should also be validated and suitable for the particular phage in order to assure that the preparations contain viable phage particles able to infect the target bacteria. Moreover, stability and pH control of the preparation is important as shown in the past by the rising problems observed when these facts were neglected (Merabishvili *et al.*, 2009).

6. Commercialization of phage-based products

Despite the good scientific results and the economic viability of phage products an important issue that cannot be forgotten is the public acceptance which can constitute a serious obstacle to the introduction of phages in food. It is likely that consumers will feel an antipathy when knowing that live viruses are being added in their foods and that will be ingested by them. First of all, phages are viruses that only infect bacteria and not eukaryotic cells as the human cells. Moreover they are very specific to the target bacteria avoiding this way dysbiosis. It is also important to note that the use of a virus to combat a pathogen is not so strange since some vaccinations are carried out using live, albeit attenuated, eukaryotic viruses. Since phages have been identified in drinking water, and foods such a yoghurt and salami they are generally considered safe (Rohwer, 2003). Also, this means not only that phages are already inside our body but also that they are constantly being ingested. Different phages, applied at different doses, using different routes of administration in humans during the long history of phage therapy in the Eastern Europe did not produced serious complications (Sulakvelidze *et al.*, 2001). Moreover, in a carefully controlled double-blind study involving ingestion of phage T4 by volunteers, no side effects were reported (Bruttin & Brussow, 2005). These facts show that phages are nontoxic to animals and plants and apparently innocuous from a clinical standpoint. Along these lines, it can be conclude that the introduction of phages in human food-chain through the usage of phages as biocontrol agents in living animals or carcases can be considered safe and may be seen as a valuable alternative to the use of antibiotics in animal production.

With respect to regulations, the introduction of a biocontrol phage product in animals and foodstuff may not be as stringent as its introduction in human therapy. The way for its introduction has been recently (2006) paved by the approval by the American Food and Drug Administration (FDA) of a mixed *Listeria*-phage preparation, ListShield (www.intralytix.com) to be used as a food additive in poultry derivatives and ready-to-eat meat. Another product based on a *Listeria*-phage, Listex P-100 (www.micreosfoodsafety.com), has received the status

"generally recognized as safe" (GRAS) to be used in all food products in 2007. Other phage-based products to control *E. coli* and *Salmonella* exist to be used at pre-slaughter (www.omnilytics.com and www.intralytix.com). The approval of such products proves the safety of phages and anticipates the development and introduction of new phage based products to be applied not only in foodstuff but also, at a long term, in animals and humans.

7. Conclusions

The emergence of multidrug-resistant bacteria has opened a second window for phage biocontrol. The recent work reviewed here shows that it has been established the proof-of-principle and evidences are more than enough to state that phage biocontrol, if well-conceived, is very effective in the treatment and prophylaxis of many problematic infectious diseases. Of particular interest is the potential that phage biocontrol has demonstrated against the global problematic multidrug-resistant bacteria. While the results of phage based products efficacy are very promising some consideration need to be taken into account. Efficient phage biocontrol requires the use of broad host range phages and administration of cocktails of phages with complementary host ranges and target receptors (showing thus cross-resistant lytic activity) in order to circumvent the emergence of phage resistant bacteria. Also, *in vivo* studies with suitable models should always be performed to assess the efficacy of the phage based product. From an economical and practical point of view, the best route of administration at an industrial scale is obviously through food and drinking water. For a successful application of phages it is important to understand the epidemiology of the pathogen against which the phage preparation is to be used in order to identify the critical intervention points in the processing cycle where phage application would be most beneficial. On the other hand, consistent pathogenic bacteria reduction is only achieved short after phage administration suggesting that phages should be applied in livestock shortly before slaughter and/or post-slaughter in carcasses and foodstuff. Besides reducing significantly the bacterial loads in the food chain with the consequent reduction in foodborne incidences it will impair the emergence of phage resistant bacteria.

Finally, although the consumers may be reluctant to the introduction of phages in the food chain, they have already shown to be safe for the environment, animals and humans with high efficiency in the reduction of foodborne pathogens. Moreover, some phage products are already commercially available and thus the way for the introduction of new phage based products is now open.

Overall, it can be concluded that phages can and should be used, not only as alternative, but also as substitutes of antibiotics and chemical antibacterials, in the control of foodborne pathogens in livestock and foodstuff.

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